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חוק הפטנטים, תשכ"ז – 1967
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אני, (שם המבקש, מענו ולגבי גוף מאוגד מקום התאגדותו)
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## תכשיר רוקחי לטיפול בליקויי הקרשת דם

(באנגלית)
(English)

## Pharmaceutical composition for the treatment of blood coagulation disorders

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Signature of Applicant

For the Applicants,
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By: —

היום This 26 בחודש of April שנת 1998 of the year

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# תכשיר רוקחי לטיפול בליקויי הקרשת דם

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# Pharmaceutical composition for the treatment of blood coagulation disorders

Opperbas Holding B.V.

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# PHARMACEUTICAL COMPOSITION FOR TREATMENT OF BLOOD COAGULATION DISORDERS

## FIELD OF THE INVENTION

The present invention relates to a stable pharmaceutical formulation for the slow release of coagulation promoting substances for the treatment of blood coagulation disorders.

## BACKGROUND OF THE INVENTION

Hemophilia A is one of the most frequently occurring inherited coagulation disorders. Patients with hemophilia A are prone to frequent hemorrhages as a result of one or more misfunctions of the coagulation system. One of the causes of hemophilia is a shortage of Factor VIII (FVIII) in the blood. This problem can be treated with Factor VIII concentrates. However, in about 15% of the patients the occurrence results of Factor VIII neutralizing antibodies, so-called inhibitors, whereby a therapy with Factor VIII concentrates is hardly possible.

Two basic approaches have been described in the literature to protect FVIII from inactivation by inhibitors.

WO/80/01456 to Hemker discloses a pharmaceutical composition suitable for oral administration comprising FVIII incorporated within liposomes of 0.5-1.0 microns formed from phospholipids. The phospholipids have a net charge, and the FVIII is incorporated between the

layers of the liposome. It is claimed that FVIII levels in the plasma remained above about 5% of the normal value for a period of 50 hours.

US 4,348,384 to Horikoshi states that a composition as described in Hemker was prepared, but did not give satisfactory results. Therefore, Horikoshi incorporates a protease inhibitor into the liposome together with FVIII, in order to protect it from proteolysis. 3% of the normal plasma levels of FVIII were obtained over a period of 6 hours.

US 5,013,556 to Woodle discloses a liposome composition for use in delivering various drugs via the bloodstream. The liposome contains between 1-20 mole percent of an amphipathic lipid derivatized with a polyalkylether. Here also, the drug compound is entrapped within the liposome. These liposome compositions are available commercially under the name of Stealth® vesicles (SUV's, small unilamellar vesicles comprised of phospholipid and polyethylene glycol (PEG) covalently bound to phospholipid).

A further problem with this approach is that liposomes having a large diameter have a short half-life. Therefore, the liposomes must be downsized under high pressure, which can affect protein activities as in coagulation factors V and VIII.

In a second approach, Barrowcliffe, T.W., *et al.* (1983) J. Lab. Clin. Med. 101:34-43 teaches that mixing FVIII with phospholipid extracted from human and/or animal brain imparts significant protection to the FVIII *in vitro*. In this approach, the phospholipid is bound to the FVIII rather than encapsulating it. Kemball-Cook, G. and Barrowcliffe, T.W. (1992) Thromb. Res. 67:57-71, teaches that a negatively-charged phospholipid surface is necessary for FVIII binding. Negatively charged phosphatidyl serine and phophatidic acid were found to be highly active in binding to FVIII, while phosphatidyl choline was inactive. However, negatively-charged

phospholipids are toxic, and those derived from brain tissue may carry pathogenic agents.

## SUMMARY OF THE INVENTION

It is an object of the present invention to provide a pharmaceutical composition comprising FVIII for the treatment of blood coagulation disorders.

It is a further object of the invention to provide FVIII in a form having an extended half-life in the bloodstream.

It is a still further object of the invention to provide a method for treating patients suffering from blood coagulation disorders, particularly hemophilia, and most particularly those having FVIII inhibitors.

The present invention thus provides a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, the particles comprising 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, the polymer carrying substantially no net charge, wherein the FVIII is not encapsulated in the colloidal particles.

The present invention is based on the surprising and unexpected finding that neutral phospholipids derivatized with a bio-compatible hydrophilic polymer can be used to bind FVIII and protect it from inhibitors in the bloodstream. This provides a significant advantage over the prior art compositions, since the phospholipids used are synthetic and non-toxic, and can therefore be used *in vivo* for therapeutic treatment. Furthermore, the liposome does not encapsulate the FVIII so that smaller sized liposomes can be used which have a longer half-life *in vivo*, since they are not removed by the reticuloendothelial system (RES).

In the present specification, the terms "*substantially neutral*" and "*substantially no net charge*" mean neither positively nor negatively charged. However, a very low measured charge within experimental error of zero is included within the meaning of the above terms.

The term "*therapeutically effective amount*" is to be understood as referring to an amount of FVIII which results in a level of FVIII in the bloodstream having a desired therapeutic effect. Such an amount can be experimentally determined by administering compositions comprising different amounts of FVIII and measuring the level in the blood at various times after administration.

The amphipathic lipid used to prepare the colloidal particles is preferably a phospholipid, and may be obtained from either natural or synthetic sources. A most preferred phospholipid is phosphatidylcholine, most preferably egg-phosphatidylcholine.

The biocompatible hydrophilic polymer may include polymers from the polyalkylether, polylactic or polyglycolic acid families. Preferably, the polymer is polyethylene glycol (PEG). The purpose of the polymer is to sterically stabilize the SUVs, thus preventing fusion of the vesicles *in vitro,* and allowing the vesicles to escape adsorption by the RES *in vivo.* The polymer will preferably have a molecular weight of between about 1000 to about 5000 daltons, most preferably approximately 2000 daltons.

The colloidal particles will preferably have a mean particle diameter of between about 0.05 to about 0.4 microns, most preferably about 0.1 microns. This is to increase their circulation time *in vivo* and prevent their adsorption by the RES. The amphipathic lipid comprises approximately 1 to about 20 mole % of the particles, preferably approximately 1-5%, most preferably 5%.

A variety of known coupling reactions may be used for preparing vesicle forming lipids derivatized with hydrophilic polymers. For

example, a polymer (such as PEG) may be derivatized to a lipid such as phosphatidylethanolamine (PE) through a cyanuric chloride group. Alternatively, a capped PEG may be activated with a carbonyl diimidazole coupling reagent, to form an activated imidazole compound. Other reactions are well known and are listed, e.g. in the aforementioned U.S. 5,013,556, whose contents are incorporated herein by reference.

The FVIII used in the composition of the invention is commercially available. It may be from a natural human source, or, preferably, it may be recombinantly prepared. Recombinant FVIII is commercially available, for example, Antihemophilic Factor (Recombinant), Kogenate, Miles Inc., Pharmaceutical Division, Elkhart, IN, U.S.A., among other suppliers.

The composition of the invention is administered parenterally, preferably iv. The prior art compositions were intended for oral use only, due to side effects caused during injection by the liposome composition. The composition of the invention, on the other hand, is not toxic by injection, apparently due to the lack of charge, among other causes. Amounts of up to 0.5gm/Kg body weight of colloidal particles according to the invention have been injected without detectable toxic symptoms. The expected dose is in the approximate range of 25-75 i.u./Kg. body weight.

Although the free form of FVIII:C has a half-life of less than 2 hours (FVIII measured by clotting activity) in mice, FVIII administered in the composition of the invention is expected to be effective for at least 24 hours, which is the period of effective activity of the coagulation promoting compound.

The effectiveness of FVIII contained in the composition of the invention may be determined by a chromogenic assay which determines FVIII activity by two consecutive steps: (1) the FVIII-dependent conversion of Factor X to Factor Xa in a coagulation-factor reagent composed of purified

components, and (2) the enzymatic cleavage of a chromogenic Factor Xa substrate to yield a chromophore which can be quantified spectrophotometrically. Under appropriate assay conditions, there exists a linear relationship between the rate of Factor Xa formation and the FVIII concentration.

## DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

Methods and Materials

1. Egg phosphatidylcholine (E-PC) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) was prepared by dissolving 2.0 gr. E-PC, 1.9 mg α-tocopherol and fluorescein-labeled phosphatidylethanolamine (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by lyophilization and the lipids reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size by extruding them through a series of polycarbonate (PC) filters (0.4 μm, 0.2 μm, 0.1 μm and 0.05 μm) using the Liposofast-Basic or Liposofast-50 extruder (Avestin) to obtain liposomes of an average size of 0.1 μm.

2. Egg phosphatidylcholine/polyethyleneglycol-phosphatidyl ethanolamine (E-PC/PEG-PE) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) and polyethyleneglycol-phosphatidyl ethanolamine (PEG-PE) was prepared by mixing 0.73 gr. E-PC, 0.185 gr. PEG-PE, 0.86 mg α-tocopherol and fluorescein labeled phosphatidylethanolamine (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by lyophilization and then reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size as described in 1 above to obtain liposomes of an average size of 0.1 μm.

3. Egg phosphatidylcholine-phosphatidyl glycerol (E-PC/PG) liposomes

A tert-butanol solution of egg phosphatidylcholine (E- PC) and phosphatidyl glycerol (PG) was prepared by mixing 0.822 gr. E-PC, 0.0924 gr. PG, 0.86 mg α-tocopherol and phosphatidylethanolamine fluorescein labeled (1:1000 lipid molar ratio) in 18ml tert-butanol.

The organic solvent was removed from the lipidic mixture by lyophilization and then reconstituted in water to 10 % w/v. The obtained liposomes were reduced in size as described in 1 above to obtain liposomes of an average size of 0.1 μm.

4. Reconstitution of the human recombinant factor VIII:

Kogenate (rFVIII formulated with human albumin, Bayer) lots 70K026 and 70K027, were used in the following examples. One vial containing about 500 IU of FVIII activity was reconstituted with 2 ml water and allowed to solubilize. 200 μl aliquots were frozen at -20$^{o}$C until use.

For the preparation of albumin-depleted Kogenate, lot # 70K027 was used. 10 vials of Kogenate were reconstituted in 20 ml water and chromatographed on a hydrophilic silica gel (3-10 μm beads). Fractions of 10ml were collected and the protein and FVIII:Ag activities were monitored. A 50 % recovery in FVIII:Ag activity was found in one peak of fractions 4-6 and another of fractions 8-14. Since the protein assay gave a peak at fractions 9-12, fractions 4-6 were pooled, aliquoted and lyophilized for further use.

5. Hemophilic mice prepared as described in Bi, L., *et al.* (1995) Nature Genetics 10:119-121, were used.

6. FVIII:Ag activity was determined using a FVIII chromogenic assay commercially available from Dade AG, Dudingen, Switzerland.

7. Preparation of composition and injection to hemophiliac mice

A liposomal aliquot was mixed with a predetermined volume of FVIII to obtain a FVIII:Ag activity of 5-10 IU/ml and rolled at RT to achieve homogeneity.

8. Groups of 5-10 hemophiliac mice were injected IV bolus through the tail vein, with 200 or 400 μl of the mixture. The mice were bled from the eye at regular time intervals (1h, 4hs, 8hs, 24hs, 32hs and 48hs) and the FVIII:Ag activities in the plasma were followed.

9. The pharmacokinetics of FVIII was determined from the results by using the RSTRIP computation software to obtain the initial FVIII:C activity ($A_0$) and the half-life time ($T_{1/2}$) of the factor in the mice blood circulation.

Examples

1. Effect of Lipid composition on the Half-Life of Factor VIII.

Liposomes of 0.05μm comprising E-PC, E-PC/PEG-PE and E-PC/PG were prepared, mixed with Kogenate in a 72:1 lipid to protein (w/w) ratio and injected into hemophiliac mice. As a control, Kogenate was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #1: Effect of lipid composition on the half-life of FVIII

| Lipid composition | $A_0$* (IU/ml) | $T_{1/2}$ (hs) | no. of mice |
|---|---|---|---|
| Control | 2.22 | 4.51 | 18 |
| E-PC/PEG-PE | 3.20 | 7.84 | 10 |
| E-PC | 1.01 | 2.33 | 10 |
| E-PC/PG | Not-detectable | not-detectable | 10 |

* $A_0$ = initial concentration of FVIII:C

It can be seen from the table that liposomes containing E-PC /PEG-PE were the most effective since both the initial FVIII activity and the half-life time were higher for this composition than for Kogenate or Kogenate-liposome mixtures where the liposomes were composed of E-PC/PG or E-PC only.

Moreover, 40% of the mice injected with free FVIII and 100% of the mice injected with FVIII /PC complex did not exhibit any recovery of FVIII chromogenic activity, while only 10% of the mice injected with FVIII/PC+PEG exhibited the same phenomena 60 min. after injection.

2. Effect of Lipid/Protein Ratio on the Half-Life of Factor VIII.

Various lipid to protein ratios in the liposome composition were obtained by mixing various aliquots of liposomes of 0.05μm comprising E-PC/PEG-PE with Kogenate. These were injected into hemophiliac mice. As a control, Kogenate was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #3: Effect of factor FVIII source on the half-life of FVIII

| source | $A_0$ (IU/ml) | $T_{1/2}$ (hs) |
|---|---|---|
| Kogenate | 2.22 | 4.51 |
| Kogenate+ SUV's | 3.36 | 8.60 |
| Baxter | 1.36 | 3.83 |
| Baxter+ SUV's | 1.08 | 4.45 |
| Omrixate | 2.35 | 3.21 |
| Omrixate + SUV's | 2.31 | 3.90 |

Mixtures containing liposomes and FVIII from Baxter or Omrixate increased the half-life of the factor by 20%, when compared with the pharmacokinetic values of the free factor, as can be seen from the above table. The half-life of factor FVIII from Kogenate, mixed with E-PC/PEG-PE liposomes was twice as long as compared with the free factor form.

4. Effect of Liposome Size on the Half-Life of Factor VIII.

Liposomes were prepared containing E-PC and PEG-PE (94:6 mol %), reduced in size to mean diameters of 0.2, 0.1 and 0.05 microns using the LiposoFast-50 extruder, mixed with FVIII concentrates in a 72:1 lipid to protein ratio and injected into hemophiliac mice. As control, FVIII concentrate (Kogenate), was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #2: Effect of lipid to protein ratio on the half-life of FVIII

| lipid/prot. (w/w) | $A_0$ (IU/ml) | $T_{1/2}$ (hs) | no. of mice |
|---|---|---|---|
| 134 | 2.26 | 3.3 | 10 |
| 32 | 1.61 | 1.91 | 10 |
| 5.3 | 3.12 | 1.64 | 10 |
| 0.89 | 2.69 | 1.5 | 10 |
| Control | 2.22 | 1.5 | 18 |

It can be seen from Table #2 that increasing the lipid/protein ratio increases the half-life time of FVIII in the blood circulation in the hemophiliac mice. The differences in the initial FVIII:C activities appear not to be related to the lipid/protein ratio.

3. Effect of different Factor VIII sources

SUVs of 0.05μm were prepared containing E-PC and PEG-PE (94:6 mol %), mixed with FVIII concentrates from various sources (Kogenate, Baxter and Omrixate) in a 72:1 lipid to protein ratio and injected into hemophiliac mice. As a control, each FVIII concentrate from the various sources was diluted in saline and injected into the mice in the same manner as the liposomal mixtures. The pharmacokinetic parameters were determined as described above, and the results are summarized in the following table:

Table #4: Effect of liposome size on the half-life of FVIII

| SUV's size | $A_0$ (IU/ml) | $T_{1/2}$ (hs) |
|---|---|---|
| control | 2.22 | 4.51 |
| 0.05 um | 0.53 | 6.54 |
| 0.1 um | 1.44 | 7.79 |
| 0.2 um | 1.78 | 9.54 |

It can be seen from the Table that all of the various sized liposomal mixtures prolonged the half-life of factor FVIII as compared with the free form of factor FVIII.

It should be emphasized that the above examples relate to a hemophiliac mouse model, and therefore, the specific ratios between PC/PC-PEG and Factor VIII and the actual dosage in the mixture thereof for the treatment of human Hemophilia disease may vary from those determined in the mice model. The parameters for preparing a composition for human use may be easily determined on the basis of the mouse data by the average skilled man of the art.

CLAIMS:

1. A pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said FVIII is not encapsulated in said colloidal particles.

2. The pharmaceutical composition of Claim 1 wherein the colloidal particle has a mean particle diameter of between about 0.05 to about 0.4 microns.

3. The pharmaceutical composition of Claim 2 wherein the colloidal particle has a mean particle diameter of approximately 0.1 microns.

4. The pharmaceutical composition of Claim 1 wherein said amphipathic lipid is a phospholipid from natural or synthetic sources.

5. The pharmaceutical composition of Claim 4 wherein said amphipathic lipid is egg-phosphatidylcholine.

6. The pharmaceutical composition of Claim 1 wherein said biocompatible hydrophilic polymer is selected from the group consisting of polyalkylether, polylactic and polyglycolic acid families.

7. The pharmaceutical composition of Claim 6 wherein said biocompatible hydrophilic polymer is polyethylene glycol.

8. The pharmaceutical composition of Claim 7 wherein the polyethylene glycol has a molecular weight of between about 1000 to about 5000 daltons.

9. The pharmaceutical composition of Claim 8 wherein the polyethylene glycol has a molecular weight of approximately 2000 daltons.

10. The pharmaceutical composition of Claim 1 wherein the FVIII is from a natural source.

11. The pharmaceutical composition of Claim 1 wherein the FVIII is recombinantly prepared.

12. The pharmaceutical composition of Claim 1 wherein the particle to FWIII ratio (w/w) is between about 30 and about 500.

13. The pharmaceutical composition of Claim 1 wherein the particle to FWIII ratio (w/w) is approximately 300.

14. Method of treatment of a patient suffering from hemophilia comprising administrating to said patient a pharmaceutical composition for parenteral administration comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said FVIII is not encapsulated in said colloidal particles.

15. Use of a colloidal particle in the preparation of a pharmaceutical composition for parenteral administration for treatment of a patient suffering from hemophilia comprising a therapeutically effective amount of coagulation factor VIII (FVIII) and substantially neutral colloidal particles, said particles comprising approximately 1-20 mole percent of an amphipathic lipid derivatized with a biocompatible hydrophilic polymer, said polymer carrying substantially no net charge,

wherein said FVIII is not encapsulated in said colloidal particles.

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